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WIP/WASp-Based Actin-Polymerization Machinery Is Essential for Myoblast Fusion in *Drosophila*

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SUMMARY

Formation of syncytial muscle fibers involves repeated rounds of cell fusion between growing myotubes and neighboring myoblasts. We have established that Wsp, the *Drosophila* homolog of the WASp family of microfilament nucleation-promoting factors, is an essential facilitator of myoblast fusion in *Drosophila* embryos. D-WIP, a homolog of the conserved Verprolin/WASp Interacting Protein family of WASp-binding proteins, performs a key mediating role in this context. D-WIP, which is expressed specifically in myoblasts, associates with both the WASp-Arp2/3 system and with the myoblast adhesion molecules Dumbfounded and Sticks and Stones, thereby recruiting the actin-polymerization machinery to sites of myoblast attachment and fusion. Our analysis demonstrates that this recruitment is normally required late in the fusion process, for enlargement of nascent fusion pores and breakdown of the apposed cell membranes. These observations identify cellular and developmental roles for the WASp-Arp2/3 pathway, and provide a link between force-generating actin polymerization and cell fusion.

INTRODUCTION

The evolutionarily conserved Arp2/3 protein complex is the primary microfilament-nucleating machinery in eukaryotic cells (Pollard and Beltzner, 2002; Welch and Mullins, 2002). To perform its diverse cellular roles, the complex must first be activated by nucleation-promoting factors (NPFs), such as members of the WASp and WAVE/SCAR protein families (Millard et al., 2004). These elements serve as essential mediators, linking signal-transduction pathways and Arp2/3-based actin polymerization (Stradal and Scita, 2006). Actin polymerization triggered by this system is translated into forces that drive a variety of key cellular functions, including cell locomotion (Pollard and Borisy, 2003), motility of membrane-bound particles within cells (Fehrenbacher et al., 2003), and formation of endocytic vesicles (Kaksonen et al., 2006).

A major challenge in the field is the assignment of physiological roles to this potent cellular machinery during the development of multicellular organisms. While genetic approaches in model organisms have shown promise in this regard (Vartiainen and Machesky, 2004), the numerous and sometimes overlapping roles assigned to the Arp2/3 system often prove difficult to separate. Our previous work has shown that Wsp, the *Drosophila* WASp homolog, acts as an Arp2/3 activator in restricted developmental contexts (Ben-Yaacov et al., 2001; Zallen et al., 2002), thus allowing for characterization of Arp2/3 function in vivo. Here, we utilize this approach to reveal an unexpected involvement of the WASp-Arp2/3 system in myogenesis. Specifically, we show that this system plays a distinct role in myoblast fusion during *Drosophila* embryogenesis.

Somatic muscle fibers in the mature *Drosophila* embryo are comprised of multinucleated cells that form by multiple rounds of fusion between two distinct myoblast subpopulations (Baylies et al., 1998; Abmayr et al., 2003; Chen and Olson, 2004). After the initial specification of the mesoderm, each embryonic trunk hemi-segment contains ~30 "founder cell" myoblasts, which will direct muscle formation and differentiation, and a large number of fusion-competent myoblasts (FCMs). Founder cells possess the information necessary for determining the identity and size of the individual somatic muscles, while the FCMs serve as a repository that will add cytoplasmic bulk to each muscle fiber (Bate, 1990; Rushton et al., 1995).

Recognition and association of founder cells and FCMs are based on heterotypic interactions between differentially expressed immunoglobulin superfamily cell-surface proteins. Founder cells express Dumbfounded (Duf) and the closely related Roughest (Rst), which serve as attractants for FCMs (Ruiz-Gomez et al., 2000; Strunkelnberg et al., 2001). Physical association between Duf/Rst and the FCM-specific protein Sticks and Stones (SNS) (Bour et al., 2000) provides a key step in myoblast adhesion and alignment of the myoblast cell membranes. Founder cells initially fuse with one or two FCMs, leading to the formation of bi-/trinuclear muscle precursors. A second, major phase of muscle growth then ensues, in which the precursor myotubes undergo successive rounds of fusion with multiple FCMs. In addition to the cell-adhesion molecules, genetic approaches have revealed a number of

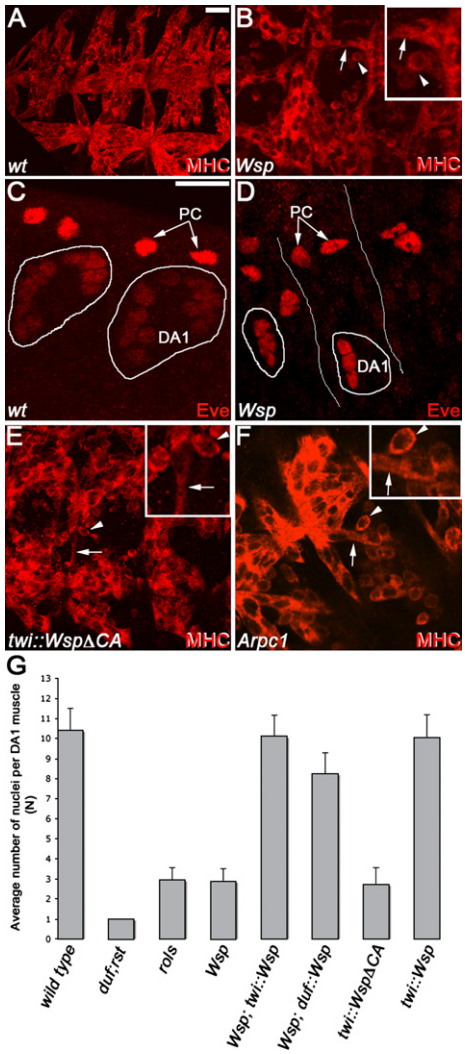

Figure 1. *Wsp* Has an Essential Function in Myoblast Fusion during *Drosophila* Embryogenesis

(A) Anti-Myosin Heavy Chain (MHC) staining (red) of musculature of a stage-16 wild-type (*wt*) embryo.

(B) Disrupted muscle pattern in a similarly staged $Wsp^{mat/zyg}$ embryo. Groups of unfused myoblasts (arrowhead) are associated with abnormally thin myotubes (arrow). Insets here and in (E) and (F) show magnified views of phenotypic aberrations.

(C) Even-skipped (Eve)-staining pattern (red) of the dorsal aspect of two neighboring segments in a stage-15 wild-type embryo. Eve is expressed in two pericardial cell nuclei (PC, arrows) and in 10–11 nuclei of the DA1 muscle (circles) in each segment.

(D) $Wsp^{mat/zyg}$ stage-15 embryos display two Eve-expressing PC nuclei in each segment, but only 2–3 Eve-positive DA1 muscle nuclei. The approximate positions of segmental borders are marked by lines.

(E) Anti-MHC staining of a stage-16 wild-type embryo expressing *UAS-WspΔCA* in muscles under the *twi-GAL4* driver. Unfused myoblasts (arrowhead) clustered along abnormal fibers (arrow) are indicated.

(F) Anti-MHC staining of a stage-16 $ArpC1^{Q25st}$ embryo shows a relatively subtle fusion phenotype.

(G) Quantification of myoblast-fusion phenotypes by the number of Eve-expressing nuclei in the DA1 muscle. The histogram shows the average number of Eve-expressing nuclei counted in individual DA1 muscles in various genotypes, including $Df(1)w^{67k30}$, which removes both *duf* and *rst* (Ruiz-Gomez et al., 2000); *Df(3L)BK9*, which removes *rols* (Menon and Chia, 2001; Rau et al., 2001); $Wsp^{mat/zyg}$ and various GAL4::UAS combinations. Error bars indicate standard deviations, and their values are noted in the text.

The scale bars represent 10 μm.

elements that contribute to various steps of the fusion process, including transcription factors, signaling molecules, and cytoskeleton-associated proteins (Chen and Olson, 2004).

The study presented here demonstrates that function of the WASp-Arp2/3 system is essential for the second phase of myoblast fusions, between maturing myotubes and FCMs, and acts after formation of fusion pores in the double membrane of the apposed cells. Recruitment of the WASp-Arp2/3 system to founder cell-FCM attachment sites is achieved via D-WIP, a *Drosophila* homolog of the Verprolin/WASp Interacting Protein (Vrp/WIP) family. Functional associations with members of this protein family constitute an evolutionarily conserved feature of WASp activity (Anton and Jones, 2006; Aspenstrom, 2005). D-WIP is specifically expressed in myoblasts and associates with the cell-surface proteins that mediate adhesion between founder cells and FCMs, thereby establishing a critical link between the cellular machineries that govern fusion and microfilament dynamics. These findings present a novel tissue context for the involvement of the Arp2/3 system in physiological events and extend the functional applications of the forces generated by actin polymerization to a central process of tissue morphogenesis.

RESULTS

Wsp Is Required for Myoblast Fusion during Embryonic Myogenesis

We followed muscle development in $Wsp^{mat/zyg}$ embryos, which completely lack *Wsp* function (Ben-Yaacov et al., 2001). Visualization of mature muscle markers, such as Myosin Heavy Chain (MHC), revealed that the muscle pattern in these mutant embryos is severely disrupted (Figures 1A and 1B). A prominent feature of the $Wsp^{mat/zyg}$ mutant phenotype is groups of mononucleated myoblasts clustered around thin, abnormally elongated fibers (Figure 1B). This phenotype is highly characteristic of embryonic muscle-fusion mutants (Dworak and Sink, 2002), suggesting that *Wsp* is required for myoblast fusion during embryonic myogenesis.

In order to quantitate the requirement for *Wsp* during myoblast fusion, we stained $Wsp^{mat/zyg}$ embryos for Even-skipped (Eve), which accumulates specifically in the nuclei of the large DA1 muscle that forms on the dorsal aspect of all embryonic trunk segments. Wild-type DA1 muscles contain 9–11 Eve-expressing nuclei (10.4 ± 1.1, n = 33) (Figures 1C and 1G), while mutants in which the fusion process is blocked express Eve in a smaller number of nuclei, corresponding to the number of fusion events that occurred (Paululat et al., 1999). The number of Eve-expressing DA1 nuclei thus serves as an established,

sensitive assay for the degree of myoblast fusion. $Wsp^{mat/zyg}$ embryos display 2–3 (2.9 ± 0.6, n = 41) DA1 nuclei per segment (Figures 1D and 1G), implying that fusion is arrested after a single round of founder cell-FCM fusion, generating a bi-/trinucleated myotube precursor.

To ascertain that the involvement of *Wsp* in muscle formation is carried out via the Arp2/3 complex, we used the mesodermal/muscle-specific driver *twist-GAL4* (*twi-GAL4*) (Baylies and Bate, 1996) to express *WspΔCA*, encoding a Wsp variant lacking the extreme C-terminal Arp2/3-binding sequence (Tal et al., 2002) (see Figure S1 in the Supplemental Data available with this article online), in wild-type embryos. We observed a strong myoblast-fusion phenotype, similar in severity to that observed in $Wsp^{mat/zyg}$ embryos (Figures 1E and 1G) (2.7 ± 0.8 DA1 nuclei, n = 31). In contrast, overexpression of full-length *Wsp* produces no deleterious effects (Figure 1G) (10.0 ± 1.1 DA1 nuclei, n = 26), underscoring the functional significance of Wsp association with Arp2/3 via the CA domain. In addition, we observed that embryos homozygous for $ArpC1^{Q25st}$, a strong mutant allele of the ArpC1 subunit (Hudson and Cooley, 2002), commonly display unfused myoblasts (Figure 1F), further implying a requirement for Arp2/3 activity during the fusion process. The relatively mild phenotype of $ArpC1^{Q25st}$ embryos (8.7 ± 1.2 DA1 nuclei, n = 21) is likely the result of maternal contribution of Arp2/3 gene products, which is essential for completion of oogenesis, and thus cannot be fully removed (Hudson and Cooley, 2002). These observations strongly imply that Wsp function during embryonic myoblast fusion involves an essential association with the Arp2/3-based actin-polymerization machinery.

The *Drosophila* Vrp/WIP Homolog *D-WIP* Is a Muscle-Specific Gene

Since WASp-family proteins commonly require activation by signaling molecules (Fawcett and Pawson, 2000), we examined the ability of Wsp variants lacking different effector-binding domains to rescue the myoblast-fusion defects in $Wsp^{mat/zyg}$ embryos (Figure S1). This analysis suggested an essential role for the N-terminal WH1/EVH1 domain, which includes the binding site for members of the evolutionarily conserved Vrp/WIP family of actin-binding proteins (Ramesh et al., 1997; Aspenstrom, 2005). A single Vrp/WIP homolog, which we refer to as D-WIP, is encoded in the *Drosophila* genome by the previously uncharacterized gene CG13503. D-WIP displays all of the structural hallmarks of Vrp/WIP homologs, including a pair of N-terminal WH2 actin-binding domains and a signature WASp-binding domain at the extreme C terminus (Figure 2A; Figure S2).

The expression pattern of *D-WIP* lends support to the notion that *D-WIP* mediates *Wsp* function in the embryonic musculature. *D-WIP* mRNA is not detected in early embryos, implying an absence of a maternal contribution (Figure 2B), while zygotic expression is first observed at stage 11 in muscle precursor cells (Figure 2C). Muscle-specific expression of *D-WIP* peaks at stage 14 (Figure 2D), corresponding to the height of myoblast fusion.

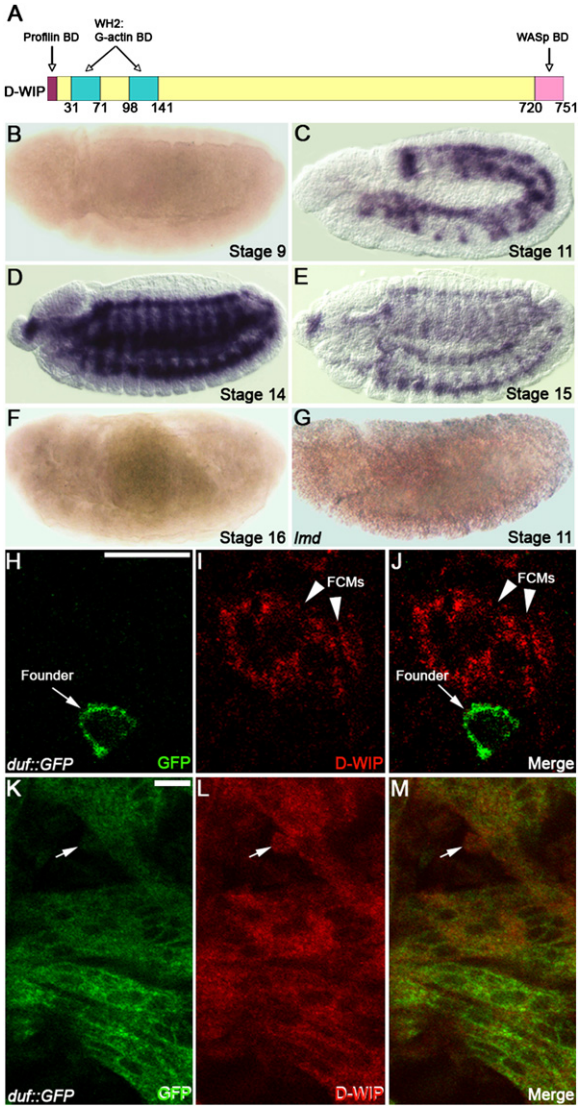

Figure 2. Myoblast-Specific Expression of *D-WIP*

(A) Schematic of the D-WIP protein, displaying the conserved structure of the Vrp/WIP protein family: N-terminal profilin-binding motifs, a pair of WH2-actin-binding domains, and a C-terminal Wsp-binding domain. Corresponding amino acid residue numbers are indicated.

(B–F) In situ hybridization to wild-type embryos with an RNA probe to *D-WIP*. Anterior is oriented toward the left, and dorsal is up. Expression is absent during (B) early stages, and is first observed at (C) stage 11 in mesodermal muscle precursors, resolving into myoblast-specific expression that peaks at (D) stage 14, then diminishes and disappears by (E and F) stage 16.

(G) *D-WIP* expression is completely missing from lmd^2 mutant embryos.

(H–J) A stage-12 *duf-GAL4::UAS-mCD8-GFP* embryo simultaneously stained with antibodies to D-WIP (red) and GFP (green). At this stage, just prior to the onset of fusion, GFP expression is restricted to founder cells (arrow), and D-WIP is restricted to the FCMs (arrowheads).

(K–M) A similarly stained stage-15 *duf-GAL4::UAS-mCD8-GFP* embryo. Low levels of D-WIP protein are now found to colocalize with GFP inside myotubes. Note that an unfused FCM attached to a myotube (arrow) expresses D-WIP, but not GFP.

The scale bars represent 10 μm.

D-WIP expression levels decrease and disappear as muscles differentiate during later stages of embryogenesis (Figures 2E and 2F).

To ascertain the identity of *D-WIP*-expressing myoblasts, we examined the expression of *D-WIP* in *lameduck* (*lmd*) mutant embryos. *lmd* encodes a transcription factor that is expressed only in FCMs and acts as a cardinal regulator of FCM-specific genes (Duan et al., 2001; Ruiz-Gomez et al., 2002). *D-WIP* mRNA cannot be detected in *lmd* mutant embryos (Figure 2G), implying that *D-WIP* expression is restricted to FCMs (see also Estrada et al., 2006).

The myoblast-subtype expression pattern of *D-WIP* was confirmed by using polyclonal rat antisera to the D-WIP protein (Figures 2H–2M). At stage 12 of embryonic development, prior to the onset of the myoblast-fusion process, D-WIP protein is distributed exclusively within the cytoplasm and subcortical regions of FCMs and is markedly absent from founder cells (Figures 2H–2J). At more advanced stages of myogenesis, however, D-WIP can be detected within growing syncytial myotubes (Figures 2K–2M). D-WIP protein, produced in FCMs, is therefore incorporated into maturing muscles after fusion of FCMs with founder cells and myotubes.

D-WIP Interacts with *Wsp* to Promote Myoblast Fusion

We disrupted the *D-WIP* locus in order to study the functional requirements for *D-WIP*. Excision of EY02177, a P element inserted in the first intron of *D-WIP* (Bellen et al., 2004), resulted in isolation of *D-WIP*^{D30}, a small chromosomal deletion uncovering the *D-WIP* gene locus, as well as five additional proximal transcription units (Figure 3A). Immunostaining of *D-WIP*^{D30} embryos with anti-MHC revealed dramatic disruption of the somatic muscle pattern (Figure 3B). As in *Wsp* mutants, many individual, unfused myoblasts, which cluster next to mispositioned muscle fibers displaying a thin, abnormal morphology, are detected. Two lines of evidence verify that the severe myoblast-fusion phenotype results specifically from disruption of *D-WIP*. Incorporating Cos1-5, a cosmid-based insertion (Kerrebrock et al., 1995; Verstreken et al., 2003) into the *D-WIP*^{D30} mutant background, restores all of the deleted genomic sequences apart from the *D-WIP* locus (Figure 3A), but embryos of this genotype continue to exhibit severe myoblast-fusion abnormalities (not shown). A complementary approach employed expression of a *UAS-D-WIP* transgene in *D-WIP*^{D30} embryos by using the mesodermal *twi-GAL4* driver, which resulted in complete phenotypic rescue (Figure 3C).

FCM clustering near myotubes and formation of myotube precursors in *D-WIP*^{D30} mutant embryos (Figure 3B) indicate proper cell-surface localization and function of the molecular machinery governing recognition and adhesion between founder cells and FCMs. This conclusion is further supported by the localization of Duf and the Duf-binding protein Rols (Chen and Olson, 2001; Menon et al., 2005) to myoblast attachment sites in *D-WIP*^{D30} mutant embryos (Figures 3D–3F).

To further characterize the *D-WIP*-fusion defect, we monitored Eve expression in DA1 muscles of *D-WIP*^{D30} embryos. On average, only 3.0 ± 0.7 (n = 36) DA1 nuclei are observed in each segment (Figures 3G and 3M). Thus, similar to *Wsp*, *D-WIP* appears to be dispensable for muscle precursor formation, but is required for subsequent rounds of fusion between growing myotubes and FCMs.

Vrp/WIP proteins bind WASp-family proteins via a conserved domain at their C terminus (Aspenstrom, 2005; Figure S2). A variant of D-WIP lacking the Wsp-binding domain (D-WIPΔC), completely fails to rescue the *D-WIP* mutant phenotype (Figures 3H and 3M). Furthermore, this construct has a strong dominant-negative effect when expressed in muscles of wild-type embryos (Figures 3I and 3M) (3.0 ± 0.5 DA1 nuclei, n = 26).

Several observations thus suggest a shared requirement for *D-WIP* and *Wsp* during embryonic myogenesis, including strong similarities in loss-of-function mutant phenotypes, and functional reliance on structural domains that mediate physical association between the two proteins. We therefore propose that *D-WIP* and *Wsp* function as a single module and act in concert to promote myoblast fusion.

The D-WIP/Wsp Module Can Function in Both Myoblast Cell Types

We next engineered conditions in which *D-WIP*/*Wsp* gene function was restricted to one of the two myoblast cell types, and monitored fusion. Supplying *D-WIP* exclusively in myotubes, by expressing *UAS-D-WIP* under control of the founder cell/myotube-specific *duf-GAL4* driver (Dutta et al., 2002), in a *D-WIP*^{D30} mutant background resulted in significant, although incomplete, rescue (Figures 3J and 3M) (8.1 ± 1.0 DA1 nuclei, n = 54). Comparable rescue of myoblast fusion in *Wsp*$^{mat/zyg}$ embryos is obtained when *UAS-Wsp* is expressed under *duf-GAL4* (Figures 1G and 3K) (8.2 ± 1.1 DA1 nuclei, n = 26).

Since FCM-specific *GAL4* drivers are not available, we adopted an alternative approach to provide *D-WIP* and *Wsp* function exclusively in FCMs. Expression in wild-type embryos of the *UAS-WIPΔC* and *UAS-WspΔCA* dominant-negative constructs via *duf-GAL4* is expected to eliminate *D-WIP*/*Wsp* activity, specifically in founder cells and myotubes. *duf-GAL4*-mediated expression of these constructs has no obvious effects on myogenesis (Figures 3L and 3M), implying that expression of *D-WIP* and *Wsp* in FCMs is sufficient for normal levels of myoblast fusion. Taken together, these results suggest that the D-WIP/Wsp system can function in both myoblast cell types during myotube formation.

D-WIP Localizes to Myoblast Fusion Sites through Association with Duf and SNS

To explore functional association of D-WIP with the myoblast-fusion machinery, we sought to determine its subcellular localization. Visualization of myoblast fusion-site components in wild-type embryos is difficult, due to the dynamic nature of the fusion process. A common solution

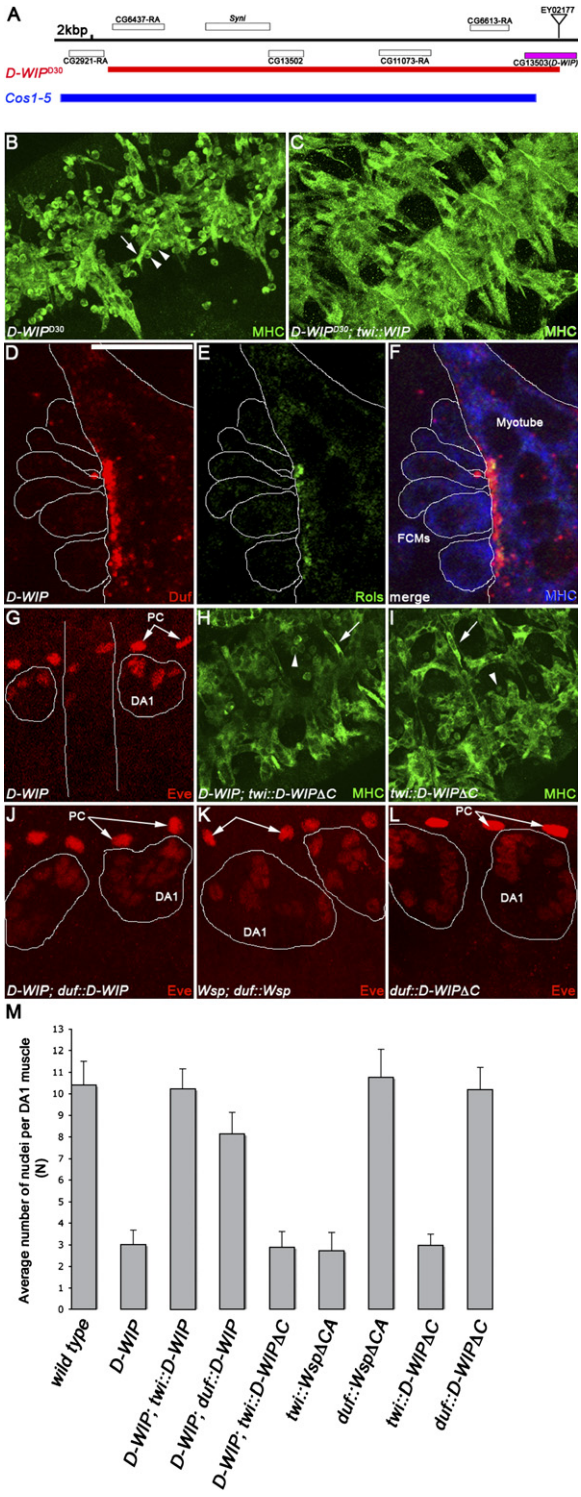

Figure 3. D-WIP Is Required for Myoblast Fusion in *Drosophila* Embryos after Formation of Muscle Precursors

(A) Scheme of the genomic region (black bar) encompassing CG13503/*D-WIP*. Position of the P[EPgy2]EY02177 transposable element used to generate the *D-WIP*^{D30} allele is indicated (triangle). White bars correspond to neighboring, proximal transcription units; those above the black bar are oriented in the 5′ to 3′ direction, and those below are oriented in the reverse orientation. Red and blue bars delineate the extent of the region deleted in the *D-WIP*^{D30} allele and the limits of the Cos1-5 genomic cosmid clone, respectively.

(B) Anti-MHC staining (green) of a stage-16 *D-WIP*^{D30} embryo reveals severe muscle-pattern defects (note the groups of unfused myoblasts [arrowheads] attached to abnormal fibers [arrow]). We note that the overall patterning of the myogenic mesoderm is unaffected in *D-WIP*^{D30} embryos (Figure S3), making it unlikely that the fusion phenotype arises as a secondary consequence of unrelated impairments to muscle development.

(C) Anti-MHC staining of a stage-16 *D-WIP*^{D30} embryo expressing *UAS-D-WIP* under *twi-GAL4* shows complete rescue of the muscle phenotype.

(D–F) Fusion-arrested myotube and attached FCMs in a stage-16 *D-WIP*^{D30} embryo stained with (D) anti-Duf (red), (E) anti-Rols (green), and (F) anti-MHC (blue). Duf and Rols localize to myotube-FCM attachment sites in the absence of *D-WIP* function. Cell outlines in these and subsequent myotube-FCM figures were drawn based on overexposed images of the MHC-staining panel. The scale bar represents 10 μm.

(G) Eve-staining pattern (red) of the dorsal aspect of a stage-15 *D-WIP*^{D30} embryo. Eve is detected in only 2–3 nuclei of the DA1 muscle (circular outlines) in each segment, indicating that, like *Wsp*, *D-WIP* is required for fusions after muscle precursor formation. Further evidence supporting this conclusion was obtained by using the founder cell/myotube marker Kruppel (Figure S3).

(H) Anti-MHC staining of a stage-16 *D-WIP*^{D30} embryo expressing *UAS-D-WIPΔC* under *twi-GAL4* demonstrates lack of phenotypic rescue.

(I) Anti-MHC staining of a stage-16 wild-type embryo expressing *UAS-D-WIPΔC* in muscles under the *twi-GAL4* driver, demonstrating a strong dominant-negative effect.

(J and K) Anti-Eve-staining pattern of the dorsal aspect of stage-15 (J) *Wsp*$^{mat/zyg}$; *duf-GAL4::UAS-Wsp* and (K) *D-WIP*^{D30}; *duf-GAL4::UAS-D-WIP* embryos. Each segment contains ~8 DA1 muscle (circles), indicating similar, partial rescue of the mutant phenotypes.

(L) Anti-Eve-staining pattern of the dorsal aspect of a stage-15 wild-type embryo expressing *UAS-D-WIPΔC* under the *duf-GAL4* driver. The number of Eve-expressing DA1 muscle nuclei is unaffected and resembles wild-type.

(M) Quantification of myoblast fusion in various *D-WIP*-related genotypes by using the DA1 muscle Eve-expression assay. Error bars indicate standard deviations, and their values are noted in the text.

is to examine localization in myoblast-fusion mutants, in which embryonic FCMs remain attached to precursor myotubes for an extended period of time (e.g., Galletta et al., 2004; Menon et al., 2005). We therefore examined *kette* mutant embryos (Figures 4A–4D) (see also Schröter et al., 2004), in which we readily observed accumulation of Duf protein along the myotube surface next to FCM attachment sites (Figure 4B). D-WIP is similarly enriched at the attachment sites, but it is present on both sides of the myotube-FCM interface (Figure 4C), an observation highlighted by the restriction of D-WIP to the FCM face of attachment sites in *mbc* mutant embryos (Figures 4E–4H).

We next turned to a cell-culture model of myoblast attachment, in which S2 cells separately transfected with *duf* or *sns* are mixed and allowed to aggregate. Colocalization of Duf and SNS at points of cell-cell contact is readily observed, mimicking in vivo myoblast attachment (Dworak et al., 2001; Galletta et al., 2004). In cells in which *D-WIP* was cotransfected with *sns* prior to aggregation with *duf*-expressing cells, D-WIP was found to colocalize

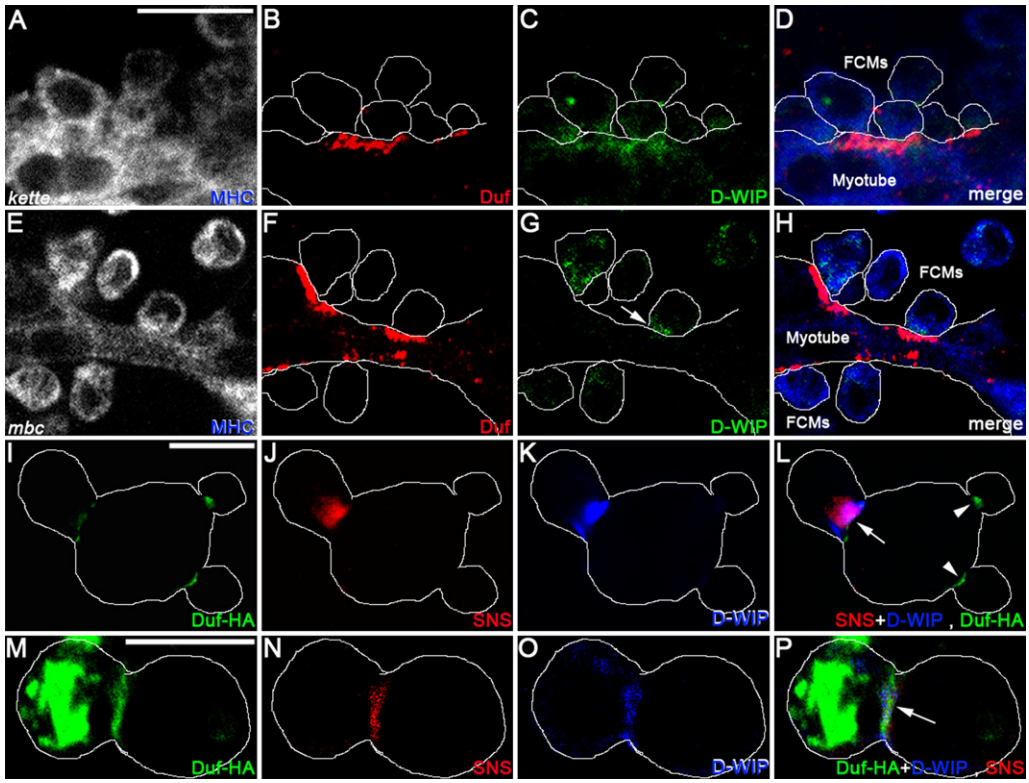

Figure 4. D-WIP Colocalizes with the Adhesion Proteins Duf and SNS at Myoblast and S2 Cell Attachment Sites

(A–D) Fusion-arrested myotube and attached FCMs in a stage-15 *kette*^{03335} mutant embryo stained with anti-MHC (gray in [A]; blue in [D]), anti-Duf (red), and anti-D-WIP (green). Duf localizes along the myotube surface of the attachment sites, while D-WIP concentrates at these sites in both myotubes and FCMs.

(E–H) Fusion-arrested myotube and attached FCMs in a similarly stained stage-15 *mbc*$^{D11.2}$ mutant embryo. The myotube is derived from a single founder cell, as even the first round of fusion fails in *mbc* embryos (Rushton et al., 1995). In this case, D-WIP localizes only on the FCM side of the attachment site (arrow in [G]).

(I–L) S2 cells coexpressing *D-WIP* and *sns* were mixed with cells expressing *duf-HA*, and aggregated cells were stained with anti-HA (green), anti-SNS (red), and anti-D-WIP (blue). D-WIP colocalizes with SNS at Duf/SNS attachment sites (arrow). Duf-expressing cells attach to each other as well (arrowheads). S2 cell outlines in these and subsequent figures were drawn based on the transmitted-light image.

(M–P) S2 cells coexpressing *D-WIP* and *duf-HA* were mixed with cells expressing *sns*, and aggregated cells were stained with anti-HA (green), anti-SNS (red), and anti-D-WIP (blue). D-WIP is again found at the Duf/SNS attachment sites, colocalizing, in this case, with Duf-HA.

The scale bars represent 10 μm.

with SNS at the cell attachment sites (Figures 4I–4L). D-WIP similarly colocalized with Duf at cell attachment sites when these proteins were coexpressed in S2 cells, followed by aggregation with *sns*-expressing cells (Figures 4M–4P). D-WIP appears to localize more avidly to the attachment sites when transfected into *sns*-expressing cells. In addition to the localization experiments, we were able to demonstrate coimmunoprecipitation of D-WIP and Duf when coexpressed in S2 cells (Figure S4). In summary, this series of in vivo, cell-culture, and biochemical approaches strongly supports an association between D-WIP and the Duf/SNS cell-surface adhesion molecules, on both aspects of myoblast attachment sites.

D-WIP Recruits Wsp to Myoblast Fusion Sites

An established function for Vrp/WIP proteins is to localize WASp to cortical sites at which Arp2/3 activity is required (Moreau et al., 2000; Sasahara et al., 2002). We sought to determine whether a similar scenario operates during myoblast fusion. To visualize Wsp in these experiments, we made use of a Wsp-GFP fusion protein that is fully functional, as determined by in vivo rescue experiments (not shown). When expressed separately in S2 cells, Wsp-GFP displays a punctate, cytoplasmic distribution (Figure 5A), while D-WIP localizes just beneath the surface of these cells (Figure 5B). Upon coexpression, however, Wsp-GFP colocalizes with D-WIP and acquires its subcortical pattern (Figures 5C and 5D). D-WIPΔC, which lacks the putative Wsp-binding domain (Figure 2A), localizes to the cell cortex, but fails to recruit Wsp-GFP, which remains cytoplasmic (Figures 5E and 5F). These results imply that D-WIP associates with Wsp through the conserved C-terminal domain and is capable of altering Wsp subcellular localization.

We utilized Duf/SNS-based S2 cell aggregation to test if D-WIP recruits Wsp to sites of myoblast attachment.

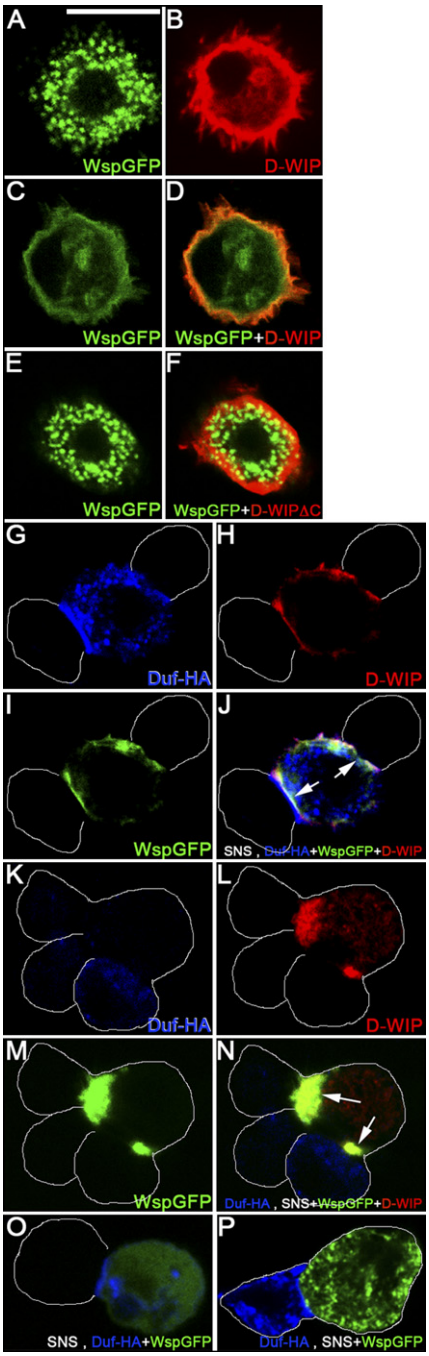

Figure 5. Wsp Is Recruited to Duf/SNS Attachment Sites in a D-WIP-Dependent Manner

(A) Wsp-GFP (green) displays a punctate, cytoplasmic distribution upon expression in S2 cells.

(B) Anti-D-WIP staining of S2 cells (red) reveals a subcortical localization of transfected D-WIP. The scale bar represents 10 μm and is applicable to all panels.

(C and D) S2 cells coexpressing *Wsp-GFP* and *D-WIP* were fixed and stained with anti-D-WIP. Wsp-GFP (green) now colocalizes with D-WIP (red) and acquires its subcortical pattern.

(E and F) S2 cells coexpressing *Wsp-GFP* and *D-WIPΔC* were fixed and stained with anti-D-WIP. Here, Wsp-GFP (green) retains its cytoplasmic distribution and is not recruited to the cortex by D-WIPΔC (red).

(G–J) Anti-HA (blue) and anti-D-WIP (red) staining of S2 cells cotransfected with *duf-HA*, *D-WIP*, and *Wsp-GFP* and aggregated with *sns*-expressing S2 cells. Both D-WIP and Wsp-GFP localize to the Duf/SNS attachment sites (arrows).

(K–N) Anti-HA (blue) and anti-D-WIP (red) staining of S2 cells cotransfected with *sns*, *D-WIP*, and *Wsp-GFP* and aggregated with *duf-HA*-expressing S2 cells. D-WIP and Wsp-GFP again localize to the Duf/SNS attachment sites.

(O and P) When the experiments shown in (G)–(J) and (K)–(N) are repeated in the absence of cotransfected *D-WIP*, Wsp-GFP retains a cytoplasmic distribution and is not recruited to attachment sites, whether cotransfected with (O) *duf-HA* or (P) *sns*.

Coexpression of *Wsp-GFP* and *D-WIP* together with *duf* in S2 cells, followed by aggregation with *sns*-expressing cells, results in the joint recruitment of Wsp-GFP and D-WIP to the Duf-SNS attachment sites (Figures 5G–5J). In a converse experiment, *Wsp-GFP* was expressed together with *D-WIP* and *sns* prior to aggregation with *duf*-expressing cells. Again, both Wsp-GFP and D-WIP are strongly recruited to the Duf-SNS attachment sites (Figures 5K–5N). In the absence of D-WIP, however, Wsp-GFP maintains its cytoplasmic distribution in either *duf*- or *sns*-expressing cells (Figures 5O and 5P).

Is Wsp similarly recruited in vivo to sites of myoblast fusion? Toward this end, *UAS-Wsp-GFP* was expressed in *kette* mutant embryos with the *twi-GAL4* driver. We found that Wsp-GFP localizes with D-WIP to both aspects of myotube-FCM attachment sites in these embryos (Figures 6A–6D). In contrast, Wsp-GFP assumes a cytoplasmic distribution in both the myotubes and FCMs of *D-WIP*^{D30} mutant embryos (Figures 6E–6H).

If the primary role of D-WIP is to recruit Wsp to sites of myoblast fusion, it may be possible to bypass the requirement for *D-WIP* by localizing Wsp to the myoblast cell surface via alternative means. Indeed, expression of *UAS-Wsp*^{myr}, which encodes a myristoylated, membrane-tethered form of Wsp (Bogdan et al., 2005) in *D-WIP*^{D30} mutant embryos, resulted in substantial rescue of the *D-WIP*^{D30}-fusion phenotype (Figures 6I and 6K) (7.4 ± 1.1 DA1 nuclei, n = 29). Expression of the *UAS-Wsp* full-length construct in *D-WIP*^{D30} mutant embryos with the same driver has no rescuing effect (Figures 6J and 6K) (2.7 ± 1.0 DA1 nuclei, n = 36), underscoring the significance of Wsp membrane localization to the fusion process. Thus, Wsp localization via D-WIP to the myotube-FCM attachment site is an essential feature of myoblast fusion.

D-WIP and *Wsp* Are Required for a Late Event in the Process of Embryonic Myoblast Fusion

Transmission electron microscopy (TEM) analysis has established that myoblast fusion in *Drosophila* embryos proceeds as a sequence of defined morphological events (Doberstein et al., 1997). Adhesion and apposition of myoblast cell membranes are followed by the appearance of vesicular and plaque-shaped electron-dense structures on both sides of the apposed membranes. Initial cytoplasmic continuity is then obtained upon formation of small (<200 nm) fusion pores linking the two cells (Figure 7A).

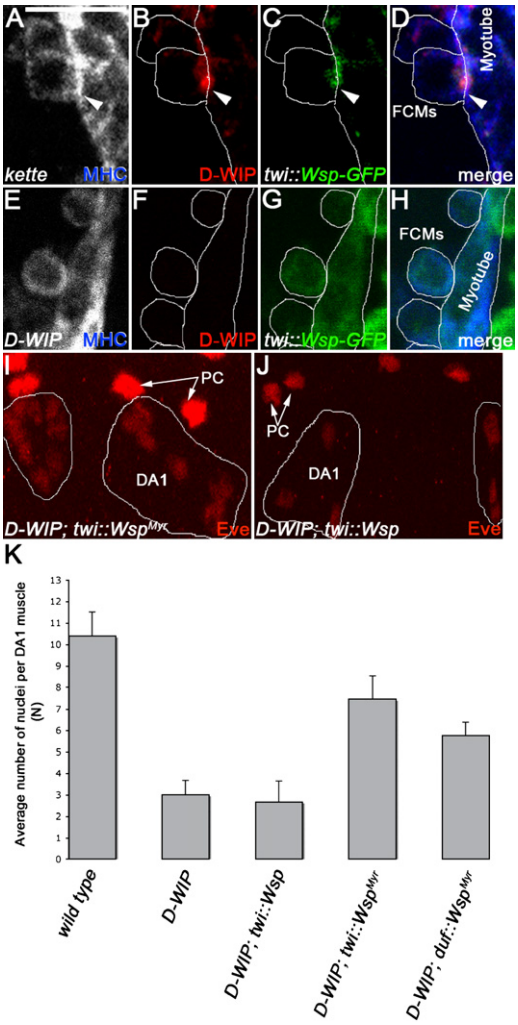

Figure 6. Wsp Is Recruited to Myotube-FCM Attachment Sites in a D-WIP-Dependent Manner

(A–D) Fusion-arrested myotube and attached FCMs in a stage-15 *kette*^{03335}; *twi-GAL4::UAS-Wsp-GFP* mutant embryo stained with anti-MHC (gray in [A]; blue in [D]), anti-D-WIP (red), and anti-GFP (green). Wsp-GFP and D-WIP concentrate on both sides of the myotube-FCM attachment sites (arrowhead). The scale bar represents 10 μm.

(E–H) Fusion-arrested myotube and attached FCMs in a similarly stained stage-15 *D-WIP*^{D30}; *twi-GAL4::UAS-Wsp-GFP* mutant embryo. In the absence of *D-WIP*, Wsp-GFP is dispersed within the cytoplasm and is not recruited to the attachment sites.

(I) Eve-staining pattern of the dorsal aspect of a stage-15 *D-WIP*^{D30}; *twi-GAL4::UAS-Wsp*^{Myr} embryo shows significant recovery of fusion, as assayed by the number of Eve-expressing DA1 muscle nuclei (circular outlines).

(J) Eve-staining pattern of the dorsal aspect of a stage-15 *D-WIP*^{D30}; *twi-GAL4::UAS-Wsp* embryo exhibits the severe *D-WIP*^{D30} fusion phenotype.

(K) Quantification of *Wsp*^{Myr} rescue by using the DA1 muscle Eve-expression assay. Error bars indicate standard deviations, and their values are noted in the text.

Vesiculation and fragmentation of the aligned double membrane ensues, and the process is completed after removal of the residual membrane material.

All fusion mutants studied to date by TEM display an arrest in the fusion process prior to the formation of pores between fusing myoblasts (Figure 7B) (Doberstein et al., 1997; Schröter et al., 2004). In contrast, TEM analysis of *D-WIP*^{D30} and *Wsp*$^{mat/zyg}$ embryos reveals a common phenotype, consistent with an exceptionally advanced stage of myoblast-fusion arrest (Figures 7C and 7D). Multiple discontinuities are apparent in the apposed myoblast membranes, suggesting that *D-WIP* and *Wsp* are not required until the final phases of double-membrane breakdown and removal. Furthermore, while the size of membrane discontinuities varies widely in wild-type myoblasts undergoing the final phase of fusion, with only a small minority (10%–20%) displaying small pores throughout the fusing membranes, we observe the latter phenotype in 50%–60% of fusing myoblasts of *D-WIP*^{D30} and *Wsp*$^{mat/zyg}$ embryos, implying that disruption of D-WIP/Wsp module function results in arrest at a discrete phase of the fusion process.

We reasoned that establishment of partial fusion between myoblasts in embryos lacking *D-WIP* and *Wsp* function would permit transfer of cytoplasmic material between the cells. A cytoplasmic form of GFP was expressed in *D-WIP*^{D30} embryos by using the founder cell/myotube *duf-GAL4* driver. "Leakage" of GFP into the attached FCMs was successfully monitored (Figures 7E and 7F). In contrast, similar analysis of *mbc* mutant embryos, in which attached myoblast membranes remain intact, failed to detect any GFP in myotube-attached FCMs (Figures 7G and 7H). These findings further substantiate the TEM analysis of the *D-WIP* and *Wsp* mutant phenotypes, and they demonstrate that *D-WIP* and *Wsp* function is required during the final stages of myoblast fusion.

DISCUSSION

D-WIP Is a Muscle-Specific Regulator of WASp

This study identified an exceptional and highly cell-type-specific mode for regulating the Arp2/3 system. Functional selectivity in this system is usually achieved via spatial and temporal control over the operation of signal-transduction pathways and the resulting production of potent activating elements for the relevant Arp2/3 nucleation-promoting factor (Stradal and Scita, 2006). In contrast, it is the restricted expression of D-WIP in the FCMs that confines Wsp-mediated triggering of Arp2/3 activity to the fusing myoblasts of *Drosophila* embryos. Transcriptional control over *D-WIP* expression, governed directly or indirectly by the Lmd transcription factor, thus provides a means for translating embryonic patterning schemes into distinct and specific cellular activities, which can profoundly influence cell morphology.

The structural basis for the interaction between D-WIP and Wsp is consistent with the established principles of Vrp/WIP-WASp protein association, which rely on an interaction between an ~25 residue long peptide from the extreme C-terminal region of Vrp/WIP proteins and the WH1/EVH1 N-terminal region of WASp proteins (Volkman et al., 2002; Aspenstrom, 2005). Most critical residues

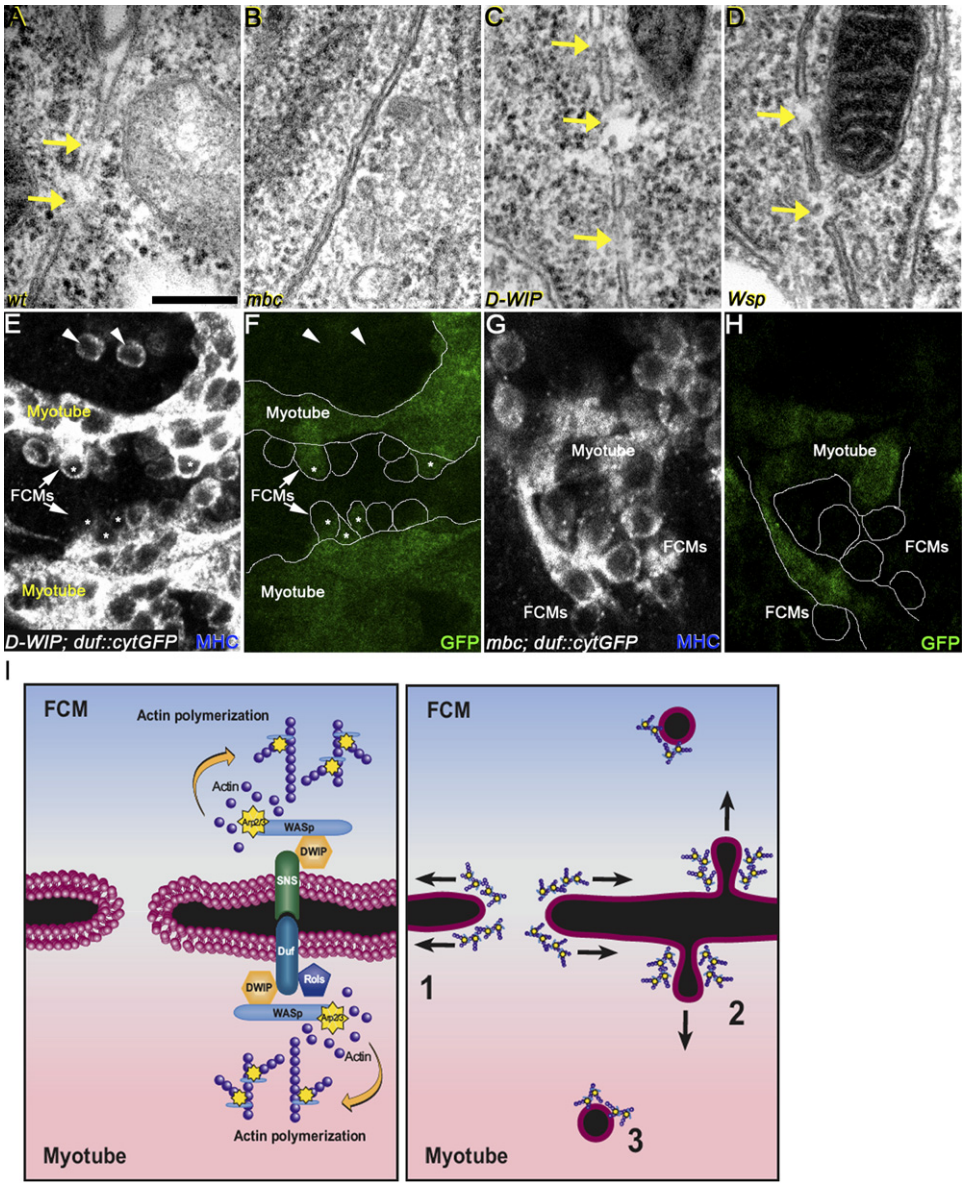

Figure 7. Fusion Pores Form and Cytoplasmic Continuity Is Achieved in *D-WIP* and *Wsp* Mutant Embryos

(A) TEM image of fusing myoblasts in a stage-13 wild-type embryo. The fusion process has entered the final phase, and small discontinuities (pores) appear in the apposed membranes (arrows). The scale bar represents 200 nm.

(B) Attached myoblasts in a stage-13 $mbc^{D11.2}$ mutant embryo. Fusion does not proceed beyond pairing and alignment of membranes.

(C) Attached myoblasts in a stage-13 $D\text{-}WIP^{D30}$ mutant embryo. Multiple, small discontinuities (arrows) disrupt the apposed membranes.

(D) Attached myoblasts in a stage-13 $Wsp^{mat/zyg}$ mutant embryo. The arrested fusion phenotype is very similar to the one observed in $D\text{-}WIP^{D30}$ embryos.

(E and F) Fusion-arrested myotubes and attached FCMs in a stage-15 $D\text{-}WIP^{D30}$; *duf-GAL4::UAS-GFP* mutant embryo, stained with anti-MHC (gray) and anti-GFP (green). Cytoplasmic GFP, expressed in the founder cells/myotubes, is often also present in FCMs (asterisks), demonstrating continuity between the myotubes and attached myoblasts. GFP is never found within unattached FCMs (arrowheads).

(G and H) Fusion-arrested myotubes and attached FCMs in a stage-15 $mbc^{D11.2}$; *duf-GAL4::UAS-GFP* mutant embryo, stained with anti-MHC (gray) and anti-GFP (green). In this case, GFP remains sequestered within myotubes, consistent with an early block in the fusion process.

(I) Models for recruitment of the Wsp-Arp2/3 system to myoblast attachment sites (left), and utilization of polymerization-derived force in myoblast fusion (right). D-WIP localizes to cell-surface regions of myotube-FCM contact and adhesion, via association with the transmembrane myoblast adhesion proteins Duf and SNS. D-WIP recruits Wsp to the myoblast contact area, providing for localized nucleation of actin filaments via the Arp2/3 complex in both myoblast types during fusion. Force generated by localized, Arp2/3-based actin polymerization is utilized after pores have formed in the apposed membranes. This force can be used for (1) enlargement of the nascent pores via interaction of branched actin networks with the perforated membranes, (2) breakdown of membranes via endocytosis, or (3) propulsion of vesiculated membrane particles, formed during the final stages of fusion, away from the myoblast attachment sites.

within these domains are conserved in the *Drosophila* homologs. Moreover, our genetic data and S2 cell localization observations strongly implicate these domains in mediating physical association between the two proteins.

By virtue of its association with the cell-surface adhesion proteins Duf and SNS, expressed in founder cells and FCMs, respectively, D-WIP may impose a common functionality on these distinct myoblast types. Yet to be determined, however, is the nature of the interaction between D-WIP and the myoblast-attachment machinery, and whether this interaction is constitutive or is dependent upon founder cell-FCM contact. Colocalization in both developing embryonic muscles and aggregated S2 cells, as well as the coimmunoprecipitation of D-WIP and Duf, underlies our suggestion of a physical association, but whether this association is direct requires further investigation. The model shown in Figure 7I summarizes the succession of protein-recruitment events that we have identified at the myoblast attachment site.

The lack of significant sequence homology between the cytoplasmic portions of the Duf and SNS proteins, and the comparatively tighter correspondence between D-WIP and SNS localizations, may be indicative of distinct modes of association between D-WIP and the two types of adhesion proteins. It is interesting to note in this context that mammalian Nephrin, which shares structural and sequence similarities with SNS (Bour et al., 2000), employs direct binding of its cytoplasmic portion to the adaptor protein Nck, as a means of establishing a functional link to the actin-based cytoskeleton (Jones et al., 2006).

WASp-family proteins are thought to reside in an auto-inhibited conformation, which prevents productive interaction with Arp2/3 and is alleviated only by binding of signaling molecules (Fawcett and Pawson, 2000). Scenarios consistent with a recruiting role for Vrp/WIP proteins have been described, including involvement of WASp in actin-based motility of intracellular pathogens (Moreau et al., 2000) and in cytoskeletal remodeling of the immune synapse (Sasahara et al., 2002). However, Vrp/WIP proteins on their own fail to stimulate, or may even inhibit, WASP-based Arp2/3 activation (Martinez-Quiles et al., 2001; Ho et al., 2004), implying a requirement for additional activating elements. Our observation that Wsp^{Myr}, a membrane-tethered form of Wsp, can partially compensate for loss of *D-WIP* function is consistent with an exclusive recruitment role for D-WIP. However, we should bear in mind that an additional step of Wsp activation may be required after its recruitment. Since the results of our phenotypic rescue experiments further imply that established activators of WASp-type proteins such as CDC42 and PIP_2 do not operate in this context, the identity of an independent Wsp activator during myoblast fusion, if one indeed exists, is currently unknown.

Harnessing WASp/Arp2/3 Activation to Generate Localized Force during Fusion

Activation of the Arp2/3 complex promotes the generation of branched networks of polymerizing actin filaments, in close proximity to both the cell surface and to internal cell membranes. The physical force liberated by this energetically favorable process can be harnessed to push against, or otherwise influence, membrane behavior (Pollard and Borisy, 2003). A key challenge stemming from our experimental observations is to identify the mechanism by which Arp2/3-based force production contributes to the progress of myoblast fusion.

The detailed TEM-level description of *Drosophila* myoblast fusion (Doberstein et al., 1997) has stipulated a series of events, including formation of pores next to sites of accumulated electron-dense material along the apposed myoblast membranes, vesiculation/fragmentation of the membranes between the pores, and removal of the residual membrane material. Our analysis of the *D-WIP* and *Wsp* mutant phenotypes demonstrates a requirement for the Arp2/3 system at a relatively late stage of the fusion process, after formation of the initial fusion pores.

Much of what is known about the mechanisms driving cell-cell (including myoblast) fusion relates to recognition and adhesion between pairs of cells and construction of initial fusion pores, while the more advanced processes of pore enlargement and the eventual establishment of full cytoplasmic continuity between the fusing cells remain mostly unexplored (Podbilewicz and Chernomordik, 2005). Our demonstration of a requirement for the cellular actin-polymerization machinery at these stages holds the promise of establishing a mechanistic basis for these late events.

We can propose several possible mechanisms for the manner by which polymerization-based forces drive fusion to completion, after initial pore formation (Figure 7I). Pore enlargement during membrane fusion poses considerable energy requirements (Chernomordik et al., 2006), which Arp2/3-based polymerization seems well suited to satisfy. The ''pushing'' forces inherent in this cellular machinery (Pollard and Borisy, 2003) can be applied to the contours of nascent fusion pores, thereby ensuring their continuous expansion. Alternatively, myoblast membranes may be broken down by vesiculation, akin to endocytosis. Detailed genetic and cellular studies have demonstrated essential roles for the Vrp/WIP-WASp-Arp2/3 machinery during endocytosis of clathrin-coated vesicles in budding yeast (Sun et al., 2006), and mechanistic interpretations of the forces involved have been put forward (Kaksonen et al., 2006). In keeping with previous discussions of these issues (Podbilewicz and Chernomordik, 2005), it is tempting to suggest that electron-dense structures, common to the contact sites of myoblasts in both *Drosophila* and vertebrate species, may provide a structural framework through which polymerization-based forces exert their influence. Finally, we can envisage a role for the Arp2/3 machinery in an even more advanced step in the fusion process, namely, the final removal of residual, vesiculated membrane material from the disrupted sites of membrane contact to create full cytoplasmic continuity.

In summary, our observations linking myoblast cell-surface adhesion proteins in *Drosophila* embryos with the WIP/WASp module suggest a mechanism through

which the conserved cellular machinery promoting force production via microfilament nucleation can be harnessed to drive muscle fiber formation to completion. Future studies will determine the finer mechanistic details of the cellular mechanism employed in this instance, and the degree to which this link can be generalized to myogenesis in vertebrate species, as well as other processes of cell fusion.

EXPERIMENTAL PROCEDURES

Drosophila Genetics

Wsp germline clones were produced (Ben-Yaacov et al., 2001) by using an *FRT82B, e, Wsp*^{1} chromosome. The *D-WIP*^{D30} mutant allele was obtained after unidirectional excision of P[EPgy2]EY02177 via P element-induced male recombination (Preston et al., 1996). Recombination was induced in males of the genotype P[EPgy2]EY02177 *bw/cn*; Δ2-3/+. *D-WIP*^{D30} was the only one of 57 resulting *cn bw* recombinant chromosomes that displayed homozygous lethality.

Molecular Genetics

Wsp constructs have been previously described (Ben-Yaacov et al., 2001; Tal et al., 2002), except for *WspΔWH1* (which bears a deletion encompassing the 145 N-terminal residues of Wsp) and *Wsp-GFP*, a fusion of GFP to the C terminus of Wsp (provided by A. Müller, University of Dundee). *D-WIP* constructs were based on PCR-amplified material with appropriate primers and the GH25793 full-length *D-WIP*/CG13503 cDNA (Stapleton et al., 2002) as template. All constructs were sequenced to ensure fidelity of the amplification and subcloning. Tagged versions used in cell-culture experiments were obtained by using the Gateway cloning system (Invitrogen) and the appropriate *Drosophila* Gateway vectors (T. Murphy, Carnegie Institution of Washington).

In Situ Hybridization

A 1.1 kbp EcoR1 fragment of cDNA GH25793 (encompassing the 3′UTR and a portion of the coding region of *D-WIP*) was used as template for transcribing an RNA probe, utilizing SP6 polymerase (New England Biolabs) and the SP6-based promoter of the pOT2 vector. In situ hybridization of this probe to embryos was performed as previously described (Melen et al., 2005).

Antibody Generation

A 1.9 kbp GH25793 cDNA fragment corresponding to residues 441–751 of the D-WIP protein was subcloned into a pRSET plasmid expression vector (Invitrogen). The 6xhistidine-D-WIP fusion protein was purified on a Ni-NTA affinity column (QIAGEN), and polyclonal antisera were obtained after injection into rats. Specificity of the antisera was demonstrated by recognition of ectopically expressed *D-WIP* and absence of staining of *D-WIP*^{D30} mutant embryos (Figure S2).

Embryo Immunohistochemistry

Embryos were processed and stained as described (Ben-Yaacov et al., 2001). Primary antibodies and dilutions used in this study include anti-β-galactosidase (rabbit, 1:1,000; Cappel, or mouse, 1:200, Promega), anti-Duf (guinea-pig, 1:250) (Galletta et al., 2004), anti-D-WIP (rat, 1:100), anti-Eve (rabbit, 1:1000) (Frasch et al., 1987), anti-GFP (mouse, 1:200; Roche), anti-MHC (rabbit, 1:500; provided by Paul Fisher, SUNY Stony Brook), anti-Rols7 (mouse, 1:1000) (Menon and Chia, 2001), and anti-Wsp (guinea-pig, 1:100) (Bogdan et al., 2005). Secondary Cy2-, Cy3-, and Cy5-conjugated antibodies against the relevant species were purchased from Jackson ImmunoResearch. Fluorescent images were collected on a Bio-Rad Laboratories Radiance 2100 confocal system.

Electron Microscopy

Staged embryos were dechorionated in bleach, fixed for 15 min on the interface of a heptane:25% glutaraldehyde/PBS mixture, devitellinized manually in PBS, then stored in 2% gluteraldehyde/0.1 M cacodylate buffer (pH 7.4) at 4°C. Subsequent steps were all carried out at room temperature. Embryos were washed in 0.1 M cacodylate buffer (pH 7.4) and postfixed for 2 hr in a 1% OsO4 solution in the same buffer. After en bloc staining in 2% aqueous uranyl acetate for 2 hr, samples were dehydrated in graded ethanol solutions and embedded in graded Epon 812. Ultrathin (70–90 nm) sections were prepared with a Leica UCT Ultramicrotome and were examined by using a Tecnai 12 transmission electron microscope at 120 kV. Images were obtained and digitized with a Megaview III CCD camera, by using AnalySIS software.

S2 Cell Culture and Immunocytochemistry

S2 cells were transiently transfected with DNA by using the ESCORT IV transfection reagent. Expression of UAS-based constructs was achieved by cotransfection with an *actin-GAL4* plasmid. Expression of RmHA3-*duf* and RmHA3-*sns* (Galletta et al., 2004) was induced by 0.1 mM $CuSO_4$ 24 hr after transfection. Aggregation of Duf- and SNS-expressing cells was performed as described (Galletta et al., 2004). Cells were adhered to polylysine-coated cover slides, and immunofluorescence was performed by standard methods. Antibodies used included anti-D-WIP (rat, 1:100), anti-HA (rabbit, 1:100; Santa Cruz Biotechnology), and anti-SNS (rabbit, 1:100) (Galletta et al., 2004).

Supplemental Data

Supplemental Data include identification of WASp domains that are required for myoblast fusion, sequence homologies of D-WIP and specificity of the D-WIP antibody, normal progression of *D-WIP* mutants through early stages of myogenesis and the initial round of cell fusion, and interactions of D-WIP with Duf and are available at http://www.developmentalcell.com/cgi/content/full/12/4/557/DC1/.

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